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Altered tubulin assembly dynamics with *N*-homocysteinylated human 4R/1N tau in vitro

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ABSTRACT

Tau isoforms promote neuronal integrity through binding and stabilization of microtubule proteins (MTP). It has been shown that hyperphosphorylation of tau contributes to Alzheimer's disease (AD) pathology and related tauopathies. However, other pathogenic modifications of tau have not been well characterized. It is well accepted that elevated level of homocysteine (Hcy) is associated with neurodegenerative diseases such as AD. As a result of *N*-homocysteinylation of lysine residues, Hcy becomes a component of proteins, as a protein–homocystamide adduct, which affects protein structure and function. Here we demonstrate that *N*-homocysteinylation of human tau (4R/1N isoform) inhibits its function via impaired tau–tubulin specific binding and MTP assembly dynamics in vitro.

1. Introduction

Elevated blood concentrations of homocysteine (Hcy) has been recognized as an independent risk factor for Alzheimer's disease (AD) [1,2]. One of the proposed mechanisms of the toxicity of Hcy is the chemical modification of proteins by homocysteine thiolactone (tHcy), a reactive metabolite of Hcy [3,4]. tHcy can be formed as a consequence of an error-editing reaction, through the activity of tRNA synthetases, in pathogenic conditions. In protein biosynthesis, the non-protein amino acid Hcy, undergoes a selectivity problem, because of its similarity to methionine, leucine and isoleucine. Indeed, Hcy enters the initial step of protein biosynthesis and forms Hcy-AMP with methionyl-, leucyl- and isoleucyl-tRNA synthetases. Misactivated Hcy never incorporate into the protein structure and instead, destroys to form activated thioester, tHcy [4]. Due to electron withdrawing activity of this thioester, the pK_a of the amine group of tHcy is 7.1, which is lower than primary amine groups in amino acids side chains. tHcy was shown to acylate ε-amino groups of lysine side chains irreversibly in a non-enzymatic process so-called "*N*-homocysteinylation", which causes loss of positive charges and introduction of thiol groups to a protein molecule as Scheme 1 [5]. As a result, *N*-homocysteinylation causes a change of the protein's physicochemical properties and an extensive impact on its biological function [3,5].

The microtubule protein (MTP) network is highly dynamic and plays a crucial role in many cellular processes such as cell division and intracellular transport [6]. MTP dynamic instability is regulated by microtubule-associated proteins (MAPs) [7]. Tau is an essential MAP of the neuronal cytoskeleton and exists as six alternatively spliced isoforms, arising from a single gene located on chromosome 17 [8]. Isoforms differ from one another by the presence or absence of a 29 or 58 amino acid insert in the N-terminal half (0, 1 and 2 N) and by the inclusion, or exclusion, of a 31 amino acid repeat, encoded by exon 10 of tau, in the C-terminal half of the protein [9]. These highly conserved repeats constitute the MTP binding region of tau; therefore C-terminal part of tau encompasses either three or four (3R and 4R) contiguous microtubule-binding repeats (MTBRs) [10]. It has been found these isoforms stabilize MTP both in vitro and in vivo and 4R tau stabilizes MTP more strongly and also overexpresses in AD condition in compare to 3R form [11,12]. In this way, the positive lysine residues in the MTBRs of tau interact with the negative C-terminal of tubulin in a sequence-specific fashion [13]. Disruption of the interaction between tau and MTP either through mutation or modification in the MTBRs would result in the release of tau from MTP. Unbinding from MTP, tau may aggregate to form paired helical filaments (PHFs) [8,14]. Thus, tau binding to MTP plays an essential role in both physiological and pathological states [15].

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Several modifications have been studied for tau protein including phosphorylation [16], acetylation [17], glycosylation [18], ubiquitinylation [19], oxidation [20], or glycation [21]. In all of these modifications, the binding potential of tau to MTP undergoes significant changes [15].

These reports beside the knowledge that lysines, in MTBR of tau, are critical to the binding to MTP, prompted us to specifically investigate the effect of tau (4R/1N) *N*-homocysteinylation on its binding activities to MTP. It is likely that tau-induced conformational states of tubulin are responsible for the assembly properties and dynamic instability of MTP; then we addressed the issue of whether *N*-homocysteinylated tau can induce assembly behavior changes of MTP.

2. Materials and methods

2.1. Materials

Ethylenebis (oxyethylenenitrilo) tetraacetic acid (EGTA), guanosine-5′-triphosphate type II-S (GTP), adenosine-5′-triphosphate (ATP), phenylmethylsulphonyl fluoride (PMSF), dithiothreitol (DTT), glycerol, $MgSO_4$, homocysteine thiolactone (tHcy), dithionitrobenzoic acid (DTNB) and taxol were purchased from Sigma (Deisenhofen, Germany). Piperazine-1,4-bis(2-ethanesulfonic acid) (PIPES) was purchased from Merck (Darmstadt, Germany). $MgSO_4$, 1 M, was added to both GTP and ATP 100 mM stock solutions, as a ratio of 1:10 (v/v). Deionized and nanopure water was used in all buffers.

2.2. Purification of tau and tubulin

The pRK-T41construct containing cDNA sequence of the human brain tau amino acid isoform (4R/1N) was kindly supplied by Dr. J. Avila (Universidad Autonoma de Madrid, Spain). The digested fragment of pRK-T41 with NdeI/EcoRI was subcloned to pET23-NHis vector, constructed from the pET-23d by adding six consecutive histidine residues to the N-terminal of the protein as Histag. For protein overexpression, pET23-NHis plasmid was transformed into BL21(DE3) competent cells and purified as described previously [22]. Concentration of tau protein was determined from the absorption at 280 nm with an extinction coefficient of 7450 cm^1 - M^{-1}, adjusted to 35 μM, and its purity estimated with SDS–PAGE (12%) Coomassie Brilliant Blue staining. For MTP assembly assays, tau samples were dialyzed against 100 mM PIPES, and 1 mM DTT at pH 6.8.

MTP was isolated from cow brain by two cycles of temperature-dependent assembly and disassembly induced by 1 mM MgGTP in PEM buffer (100 mM PIPES, 1 mM EGTA, 1 mM $MgSO_4$, 1 mM PMSF and 1 mM MgATP, pH 6.9). To obtain MAP-free tubulin, protein sample was applied to a P11 phosphocellulose column (Whatman, Piscataway, NJ) and eluted tubulin fractions were stored at −70 °C within two weeks. The resulting tubulin was essentially pure (>98%) as determined by SDS–PAGE (10%), Coomassie Brilliant Blue staining. Tubulin concentration was assessed by the Bradford reagent (Bio-Rad Laboratories, Hercules, CA), using serum albumin as standard and justified to 25 μM for further experiments.

2.3. Preparation of N-homocysteinylated tau

Tau (35 μM) was extensively dialyzed against phosphate buffer (50 mM and pH 7.4) at 4 °C for 24 h prior to modification. *N*-homocysteinylated tau (*N*-Hcy-tau) was prepared by incubation of tau protein with increasing tHcy final concentrations (0.15, 1.5 and 15 mM) at 37 °C for 18 h [23]. Unreacted tHcy and hydrolysis products were removed by ultrafiltration through a 10 kD cutoff membrane (Millipore, Billerica, MA) at 4 °C.

2.4. Quantification of sulfhydryl groups

After treatment of *N*-Hcy-tau species with 5 mM DTT for 10 min and at room temperature, they were ultrafiltrated through a 10 kD cutoff membrane (Millipore, Billerica, MA). The extent of modification was quantified by assaying for free sulfhydryl groups using DTNB. The increase in absorbance at 412 nm, due to thionitrobenzoate (TNB) release, was calculated using the extinction coefficient of 13600 cm^{-1} M^{-1} [24].

2.5. MTP binding and assembly assays

Ability of modified tau to bind to MTP was examined by incubation of *N*-Hcy-tau species (10 μM) with taxol-stabiliazed MTP (25 μM) in assembly buffer (100 mM PIPES, 1 mM EGTA, 1 mM $MgSO_4$, 1 mM DTT, 1 mM MgGTP, pH 6.9) for 30 min at 37 °C. Following a 20 min ultracentrifugation, aliquots of supernatants (tau not bound to MTP) and pellets (tau bound to MTP) were subjected to SDS–PAGE and stained with Coomassie Brilliant Blue. Density of individual bands was estimated by 1-D Total Lab TL120 software (Nonlinear Dynamics Ltd., UK).

To study the effect of modified tau to promote MTP assembly, *N*-Hcy-tau species (10 μM) was incubated with tubulin (25 μM) in assembly buffer for 15 min at 37 °C. The assembly of tubulin into MTP was detected over time by measuring the turbidity at 350 nm using a Cary (Varian, AU) UV/vis spectrophotometer.

2.6. Determination of tubulin critical concentration

To determine the effect of modified tau on the critical concentration (C_c) and unassembled fractions of tubulin (F_i), tubulin (25 μM) was assembled in the presence or absence of unmodified or *N*-Hcy-tau species (10 μM) in the assembly buffer for 30 min at 37 °C. At the steady state, aliquots of the sample were diluted to different final concentrations of tubulin (C_t), followed by re-achievement of the steady state and separation of unassembled tubulin from MTP by ultracentrifugation. After passing through a P11 phosphocellulose column (Whatman, Piscataway, NJ), the tubulin concentration in the supernatant (C_s) was determined by the Bradford reagent (Bio-Rad Laboratories, Hercules, CA), using serum albumin as standard and C_c and F_i were calculated as the intercept and slope of a plot of C_s versus C_t, respectively [25].

2.7. Electron microscopy

Aliquots from assembly buffer were placed on 200 mesh grid, coated with Formvar-carbon, and stained dropwise with 1.0% uranyl acetate. The air dried samples were examined in a Philips EM208 transmission electron microscope, operated at 90 kV.

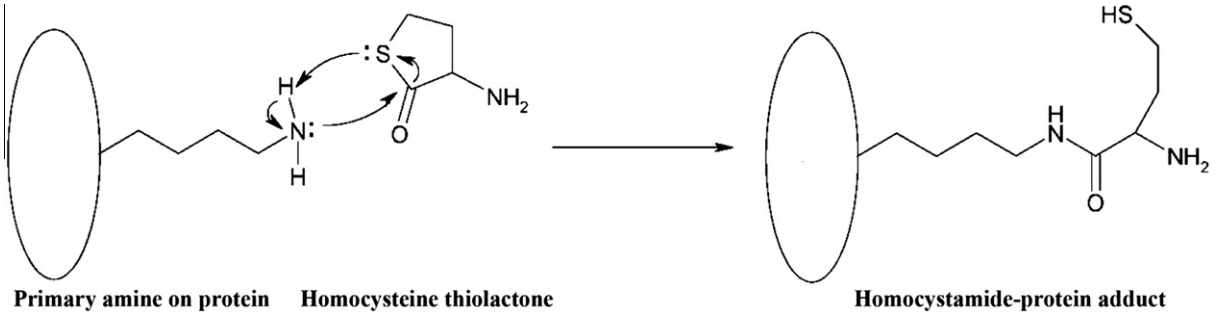
2.8. Statistics

Each experiment was carried out in triplicate and values are the means and standard deviations of three experiments. Results were analyzed for statistical significance using a two tailed student's *t* test. Changes were considered significant at *P* values less than 0.05. All statistics were performed with Sigma Plot Version 12 (Systat Software Inc., CA) and presented as the mean ± SD.

3. Results and discussion

Alteration of functional properties of proteins can be a consequence of pathophysiological accumulation of reactive metabolites that have the potential to induce protein modifications.

Tau isoforms are intrinsically basic, unfolded proteins and 4R/1N isoform contains 43 lysine residues. Net charge and isoelectric



Scheme 1. Mechanism of *N*-homocysteinylation and incorporation of thiol group into proteins.

Table 1
Molar formation of homocystamide adducts upon treatment of tau (4R/1N) with tHcy.

tHcy (mM)	–Homocystamide/tau (4R/1N) molar ratio
0	0.09 ± 0.01
0.15	1.64 ± 0.17
1.5	7.18 ± 0.84
15	25.20 ± 0.13

Stoichiometry of homocystamide adducts formation by tHcy. The determination was performed by DTNB, chromogenic measuring of the increase of absorbance at 412 nm, and by calculation with the extinction coefficient of 13600 cm^{-1} M^{-1}.

point of tau isoforms are the key factors for binding to MTP and modulating the assembly dynamics [26]. Due to the overexpression of four-reaped tau isoforms in pathological conditions and solvent accessibility of their lysines, this isoforms are more susceptible to *N*-homocysteinylation.

The malfunction of tau in nervous system appears to be likely related to its MTP interaction. Therefore, we planned to investigate the contribution of tau 4R/1N *N*-homocysteinylation in this process. For assaying the molar formation of homocystamide–tau adducts, the increase in protein thiol groups was monitored with respect to control tau. To avoid extensive oxidation of new thiol groups in *N*-Hcy-tau species, we performed the assays using DTNB, after treatment the samples with DTT (Table 1). This finding was supported by site-specific mass spectrometry analysis (unpublished data).

Kinetic stabilization of MTP, is pivotal for its physiological roles such as maintenance of axonal transportation [6]. This stabilization widely depends on efficient binding of MAPs [26]. Binding ability of tau isoforms to MTP can be partially inhibited by missense mutagenesis or modifications of lysine residues, particularly in MTBRs [27]. To assess the significance of tau *N*-homocysteinylation, we determined the ability of *N*-Hcy-tau to binding to MTP. Co-sedimentation studies of tau and taxol-stabilized MTP showed that the degree of *N*-homocysteinylation influences the ability of tau to bind to MTP. The distribution of *N*-Hcy-tau between the pellet (bound to MTP) and supernatant (not bound to MTP) after ultracentrifugation, was markedly different from that observed with the unmodified tau (Fig. 1). In particular, a large fraction of *N*-Hcy-tau was recovered in the supernatant in a dose dependent manner of tHcy, indicating that it was not bound to MTP. This suggests that *N*-homocysteinylation of tau results in a reduced affinity of tau for the MTP (see Table 2).

Native tau binds to MTP, but more importantly, it regulates nucleation and assembly characteristics of tubulin. Many studies have been performed on the ability of abnormal tau to regulate MTP nucleation and assembly processes [28]. We therefore asked

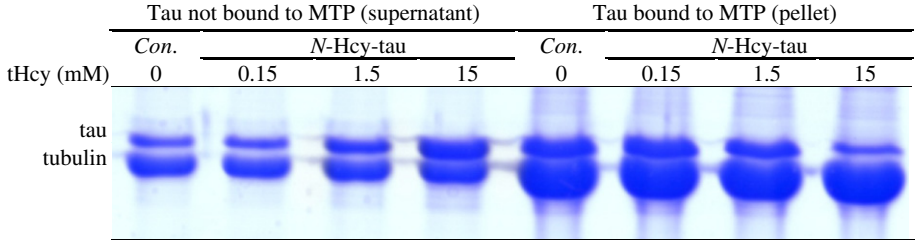


Fig. 1. MTP binding assay of unmodified tau (Con.) and *N*-Hcy-tau species. Following incubation of *N*-Hcy-tau species (10 μM) with taxol-stabilized MTP (25 μM) for 30 min at 37 °C, samples were precipitated into pellet and supernatant and subjected to SDS–PAGE. Similar results were obtained in three independent assays. A typical experiment is shown here.

Table 2
Densitometric analysis of co-sedimented N-Hcy-tau with taxol-stabilized MTP.

tHcy (mM)	Tau not bound to MTP (supernatant)				Tau bound to MTP (pellet)			
	Con.	*N*-Hcy-tau			Con.	*N*-Hcy-tau		
	0	0.15	1.5	15	0	0.15	1.5	15
Tau density%	28.86	30.99	65.27	86.11	71.14	69.01	34.73	13.89
Tubulin density%	19.37	20.65	20.02	22.13	80.63	79.35	79.98	76.87

N-Hcy-tau and tubulin densities (supernatant + pellet)%. SDS–PAGE gels were quantitated using 1-D Total Lab TL120 software (Nonlinear Dynamics Ltd., UK). The average densities of bands were measured in pixel concentration and are given in percentage of the bounded and unbounded for *N*-Hcy-tau species and assembled and unassembled for tubulin. Samples were run in triplicate.

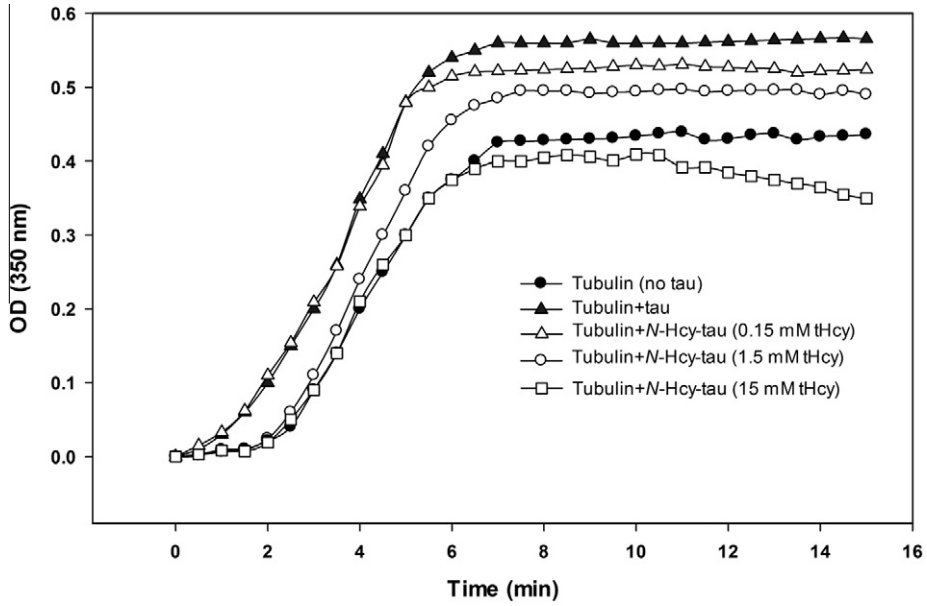


Fig. 2. MTP assembly assay in the presence or absence of unmodified tau or *N*-Hcy-tau species. MTP assembly, as determined by turbidimetry at 350 nm, was performed in the absence or presence of unmodified or *N*-Hcy-tau species. Tubulin (25 μM) was incubated without or with unmodified tau or *N*-Hcy-tau species (10 μM) and polymerized in assembly buffer to steady state at 37 °C.

the question, whether the tendency of binding *N*-Hcy-tau to MTP, correlated with the impact on nucleation and assembly dynamics. To investigate this possible effect, we monitored the raise of turbidity at 350 nm, as a probe of MTP polymerization behavior in vitro, in the absence or presence of unmodified or *N*-Hcy-tau species (Fig. 2). The lag phase, which is strongly related to nucleation efficiency [29], was shortened in the presence of unmodified and *N*-homocysteinylated (0.15 mM tHcy) tau (from 106 ± 3.04 to 49 ± 4.18 and 48 ± 2.27), indicating the promotion of MTP nucleation. In contrast, *N*-Hcy-tau (1.5 and 15 mM tHcy) failed to enhance MTP nucleation, similar to the negative control (no tau).

A short lag phase and steep rise in OD_{350}, which is proportional to the initial rate, are usually related to one another. The reason is that, in principal, a short lag phase indicates efficient nucleation. This in turn, means a specific binding of tau to tubulin, and since this is one of the main factors determining the assembly rate. Unmodified and *N*-homocysteinylated (0.15 mM tHcy) tau enhanced the initial rates in a similar manner, whereas, these rates in the presence of *N*-Hcy-tau (15 mM tHcy) and tubulin alone (no tau) were significantly low. Final plateau at steady state associates with total polymer mass [29]. Obviously, there are significant differences in the population of MTP assembled in the

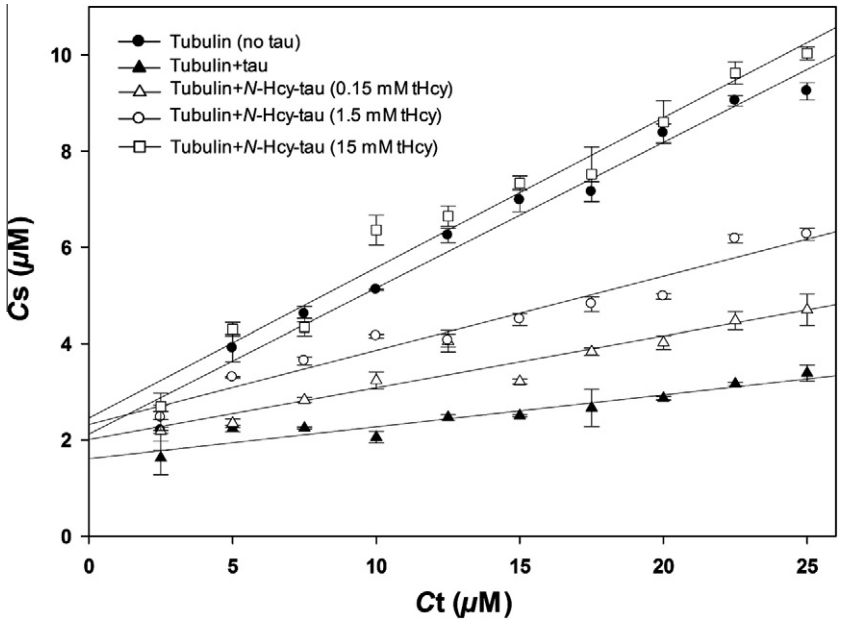


Fig. 3. Determination of the role of unmodified tau or *N*-Hcy-tau species in the critical concentration (C_c) and unassembled fractions of tubulin (F_i). Preassembled MTP samples were pre-achieved to steady state at different total tubulin concentrations (C_t) and separated from unassembled tubulin in the supernatant (C_s). C_s is plotted against C_t, giving an apparent C_c as the intercept and F_i as the slope. Each point is the average of at least three independent measurements and the lines are the results after a linear curve fitting processing for each group.

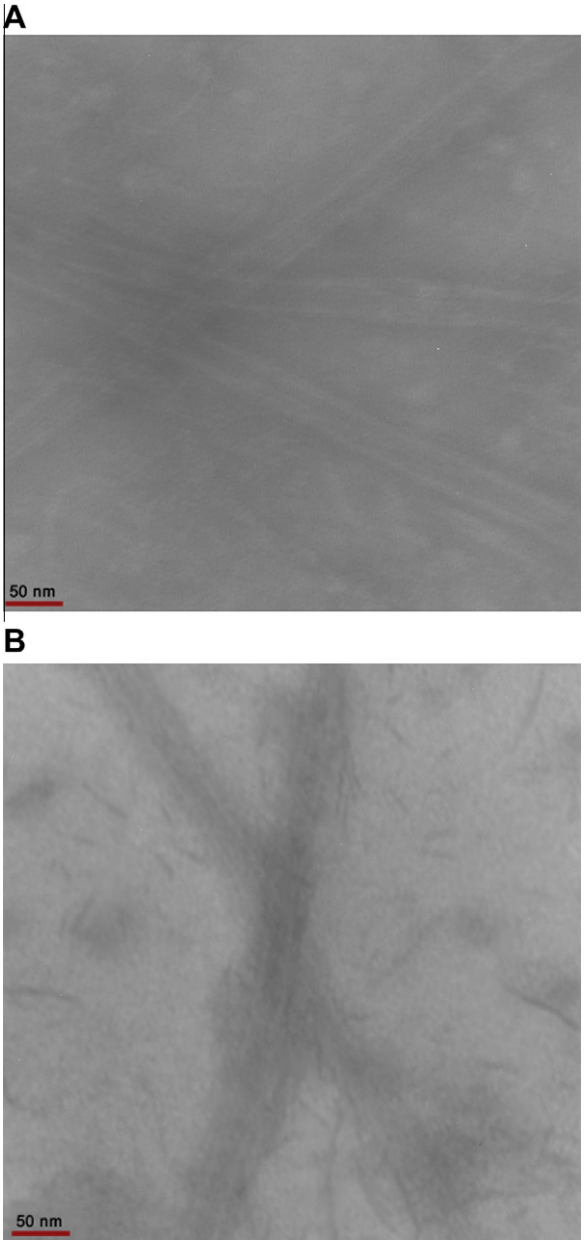


Fig. 4. Electron micrographs of unmodified and *N*-homocysteinylated tau-induced MTP assembly. MTP polymerized in assembly conditions as described in Section 2 in the presence of (A), unmodified tau (B), *N*-Hcy-tau (15 mM tHcy) and negative stained at steady state. The bar is corresponds to 50 nm.

presence of the various tau species. Upon increasing the concentration of modifier, we observed a gradual decrease of the OD_{350}. Extent of assembly decreased by 7% for *N*-Hcy-tau (0.15 mM tHcy), 12.5% for *N*-Hcy-tau (1.5 mM tHcy) and 28% for *N*-Hcy-tau (15 mM tHcy), to compare with unmodified tau. In addition, for *N*-Hcy-tau (15 mM tHcy), we observed a destabilizing effect on the MTP lattice after reaching the steady state.

These in vitro findings show that *N*-homocysteinylation of tau affects its functional activity, which is critical for binding and stabilization of MTP. Our results confirmed the importance effect of lysine modifications in MTP assembly and dynamics [17]. It has been reported that K280, which is located at MTBR (^{275}VQIINKK^{281}), is critical for binding to MTP [30]. Our unpublished mass spectrometry data revealed that in the presence of molar excess of tHcy (15 mM), K280 undergoes the modification. Then, impaired binding of tau to MTP, especially in the presence of *N*-Hcy-tau (15 mM tHcy), could be attributed to modification of this lysine group.

Tau increases the rate of MTP elongation by decreasing tubulin dissociation during assembly. This lowers the critical concentration for elongation [31]. To learn how *N*-homocysteinylation of tau affects MTP assembly, we determined the critical concentration of tubulin. To achieve this, various concentrations of preassembled tubulin (C_t) are plotted as a function of unassembled tubulin (C_s) in the supernatant, in the presence or absence of unmodified or *N*-homocysteinylated tau species (Fig. 3). We found that in the presence of *N*-Hcy-tau, critical concentration increased from 1.95 ± 0.18 μM (0.15 mM tHcy) to 2.42 ± 0.31 μM (15 mM tHcy), suggesting a functional deficit of tau caused by *N*-homocysteinylation. Also, according to earlier reports, the main effect of tau is to stabilize MTP against disassembly and cooperation to involvement of more tubulin into the MTP lattice [32]. Due to this fact, the fraction of disassembled tubulin, in the presence of native tau, is less than this fraction for tubulin alone. Our results show that this fraction increases upon tau *N*-homocysteinylation and is dependent on the state of tau modification.

The change in absorbance at 350 nm is characteristic of the formation of MTP polymers [33], but it is not clear what exact structures are being formed, particularly in the presence of high level *N*-homocysteinylated tau (tHcy 15 mM). Then, we decided to investigate the morphology of the structures formed in the reaction mixtures. To this aim, we carried out the negative staining electron microscopy at steady state of MTP assembly, in the presence of unmodified or *N*-homocysteinylated (tHcy 15 mM) tau. A typical and normal MTP were seen in assembly promoted by unmodified tau (Fig. 4A), but high level *N*-homocysteinylated tau (tHcy 15 mM) disrupted MTP assembly after reaching steady state (Fig. 4B), in consistence with time dependent decrease in absorbance after reaching this state (see Fig. 2). Disassembled and/or unassembled MTP protofilaments and ribbons, which are characteristics of disrupted and unstable MTP, were observed in Fig. 4B. Based on the MTP assembly results (Fig. 2), the initial phases of assembly, for both tubulin alone and *N*-Hcy-tau (15 mM tHcy), are similar. It seems that there isn't any specific interaction between *N*-Hcy-tau (15 mM tHcy) and tubulin in the initial steps of assembly, but, in the case of absence of any interaction, the last phase and steady state of assembly should be the same as tubulin alone. According to the destabilizing effect of modified tau (Fig. 2) and perturbation of MTP (Fig. 4B), it could be deduce that *N*-Hcy-tau (15 mM tHcy) interacts with MTP but not in a specific manner. This un-specificity could be due to the interaction of unmodified lysine groups (around 18) with tubulin C-terminals in the lattice. Therefore, we can conclude that modification of tau with high level of tHcy interferes with the normal activity of tau by abrogating the ability of this protein to stabilize MTP, and *N*-homocysteinylation is sufficient to induce this loss of function.

Axonal transport mechanism, in central nervous system, relies on MTP as routes and tracks that are stabilized by tau isoforms. This implies that the ability of tau to maintain MTP tracks seems to depend on the lack of pathogenic modifications at MTBRs. In this context, we presented distinct differences in MTP assembly parameters in vitro, depending upon the *N*-homocysteinylation status of human tau (4R/1N). Our in vitro results indeed, show that *N*-homocysteinylation of tau at critical amino acids decreases the binding ability of tau to MTP, leading to the instability of MTP and impaired its ability to promote MTP assembly. To our knowledge, these results are the first to demonstrate the significance of this type of modification on tau critical roles. While, as reported here, the *N*-homocysteinylation state of tau had a significant effect on the binding and MTP assembly behaviors in vitro, the effect in vivo has yet to be examined.

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